Isolation of endothelial cells and their progenitor cells from human peripheral blood

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Purpose: We have developed techniques to isolate endothelial cell (EC) progenitors from human peripheral and umbilical cord blood.
Methods: Human adult peripheral and umbilical cord blood monocytes were isolated by centrifugation, and progenitor cells were separated with the use of magnetic polystyrene beads that were coated with a monoclonal antibody specific for the CD34 cell–membrane antigen. Cells were propagated in selective media, and developing cultures were immunostained for CD31, CD34, factor VIII, and vascular endothelial growth factor cell receptors. ECs that developed were transfected with a gene for prourokinase and used to line ePTFE grafts, which were evaluated in vitro in a pulsatile flow system.
Results: Umbilical cord monocyte cultures demonstrated colonies that resembled ECs at approximately 2 weeks, with growth being best supported by EC growth media plus 20% calf serum with iron. Immunostaining of colonies was positive for CD31 and factor VIII. After 18 days in culture, $CD34^+$ cells from adult peripheral blood were noted, which had the typical cobblestone appearance of ECs and immunostained positively for CD31 and factor VIII–related antigens. Cultures of umbilical cord–derived cells and adult peripheral blood–derived cells developed complex line formations within 1 week in culture that stained positively for vascular endothelial growth factor receptor-2. Urokinase-transfected ECs were shown to overexpress urokinase. Prosthetic grafts lined with transfected cells showed 87.33% ± 4.97% cell adherence after 2 hours in a pulsatile flow system at clinically relevant shear stress.
Conclusion: We conclude that endothelial progenitor cells can be isolated from human adult peripheral and umbilical cord blood and developed into EC cultures as a source of cells for vascular graft seeding and gene therapy. (J Vasc Surg 2000;31:181-9.)

Despite recent advances in vascular cell biology, patency of small-diameter vascular grafts remains poor, and there are no acceptable prosthetic grafts for vessels 4 mm or less in diameter.[1] The use of endothelial cells (ECs) to line a synthetic graft has shown promise in animal models in the improvement of patency rates, and genetic modification of these cells could be of further benefit.[2]

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Effective application of these techniques in patients, however, will require an easily accessible source of ECs. One possible source of EC is cultures grown from ECs or their progenitor cells circulating in peripheral blood, which would eliminate the need for surgical removal of veins for cell harvest. We have developed methods to isolate these cells from human umbilical cord and adult peripheral blood that provide culture conditions to promote the development of ECs for use in seeding prosthetic grafts and for gene therapy.[3,4]

METHODS

Isolation of umbilical cord blood ECs. Umbilical cord blood (15-30 mL/sample) was obtained from 63 patients shortly after delivery and was placed into heparinized tubes. The blood was collected with minimal handling of the vessel. The sample was diluted 1:1 with phosphate-buffered saline solution, added to a Ficoll gradient, and cen-

trifuged at 1400*g* for 10 minutes. The buffy layer was removed and diluted 1:1 with phosphate-buffered saline solution and centrifuged at 400*g* for 7 minutes. The pellet was resuspended in Modified Dulbecco's Basic Salt Solution and centrifuged again at 400*g* for 7 minutes. This procedure was then repeated two more times. The cell pellet was resuspended in media and placed in T-25 flasks (Corning, Cambridge, Mass), coated with a matrix, and incubated at 37°C, 97% humidity, and 4.5% carbon dioxide. Twenty-one of the 63 samples were divided and cultured under more than one matrix / media condition for a total of 88 experimental trials. Three different culture matrices were used: gelatin (2 mg / mL), fibronectin (0.1 mg / mL), and fibronectin / gelatin (0.05 mg / mL, 1 mg / mL). Cell counts were not performed on mononuclear preparations isolated from umbilical cord blood. In each case, the entire mononuclear preparation was divided between several flasks at approximately equal cell density.

Five different media were initially used for the cord blood monocyte cultures: Dulbecco's modified eagle media (DMEM) plus 20% calf serum with iron; Rosewell Park Memorial Institute (RPMI) plus 10% calf serum with iron; RPMI plus 10% calf serum with iron and basic fibroblast growth factor (bFGF); EC growth media plus 20% calf serum with iron[4]; and EC growth media without serum plus bFGF. For all cultures, one half of the media was changed three times per week.

Isolation of human peripheral blood EC. The experimental protocol was approved by the William Beaumont Hospital Human Investigation Committee, and all subjects gave informed consent before blood was drawn. Approximately 50 mL of human peripheral blood from 5 healthy adult (1 female and 4 male) volunteer donors (aged 20 to 52 years) was drawn from the cephalic vein at the antecubital fossa into tubes containing 100 USP sodium heparin. The initial portion of the drawn blood was discarded to eliminate possible contamination of the sample with cells released from the vessel wall during the needle stick. After being mixed with an equal amount of isolation buffer, mononuclear cells were isolated with the use of a Ficoll gradient and centrifugation at 800*g* for 20 minutes. The buffy coat was harvested from the gradient. Uniform, magnetic, polystyrene beads (Dynal, Lake Success, NY), which were coated with a monoclonal antibody specific for human CD34, were used according to manufacturer protocol to separate endothelial progenitor cells and any $CD34^+$ ECs. Cells were incubated with beads at ratios that ranged from 0.5 beads per cell to 5 beads per cell; most experiments were performed at 1 bead per cell. Beads were separated from the suspension with a Magnetic Particle Concentrator (Dynal). Selected $CD34^+$ cells were detached from the magnetic beads by use of an antibody against the Fab portion of the monoclonal antibody to CD34. $CD34^-$ cells were isolated from the remaining suspension by centrifugation. Each cell population was then resuspended in EC growth media plus 20% calf serum with iron. After the harvest, cells were placed for culture into T25 flasks coated with a matrix of 2 mg / mL gelatin or 0.1 mg / mL fibronectin; some cells were placed in matrix-coated chamber slides or 35-mm dishes for immunostaining. Cultures were incubated at 37°C, 97% humidity, and 4.5% carbon dioxide. One half of the culture media was changed three times per week. Typically, cells were passaged when they reached 30% to 50% confluence. All experimental data reported is for cells from passage number 3 or below. Two human peripheral blood samples were subjected only to the gradient centrifugation method used in isolating umbilical cord monocytes without the use of polystyrene beads for $CD34^+$ selection and then placed in culture (as mentioned earlier).

Immunostaining. Cell line formations and colonies that appeared in umbilical cord monocyte cultures were identified by immunostaining for vascular endothelial growth factor receptor-2 (VEGF-R; Santa Cruz Biotechnology, Santa Cruz, Calif), factor VIII vonWillebrand–related antigen (Shandon Lipshaw, Pittsburgh, Pa), and CD31 (Dako Corporation, Carpinteria, Calif) and by the incorporation of 1,1´dioctadecyl-3,3,3´,3´-tetramethylindo-carbocyanine perchlorate acetylated low density lipoprotein (DiI-Ac-LDL). Once cell colonies were identified in peripheral blood cultures from $CD34^+$ cells, immunostaining was performed for factor VIII, VEGF-R, CD31, and CD34 (Novocastra Laboratories, Vector Laboratories, Burlingame, Calif). A double-staining technique was used for simultaneous staining of factor VIII and VEGF-R (Zymed Laboratories, San Francisco, Calif). Both umbilical cord and peripheral blood cultures were periodically checked for contamination with smooth muscle cells or fibroblasts, and cells with suspect morphologic features were removed with a rubber policeman. Cultures of these cells were immunostained with a monoclonal anti-human smooth muscle cell actin antibody (Shandon Lipshaw) to assess possible contamination.

Transduction of EC with preprourokinase and lining of prosthetic grafts. EC were transfected with a retroviral vector of the LN series that contained the gene for human prourokinase and

selected in medium containing G418, as previously described.[3,4] Urokinase production of transduced cells relative to nontransduced control ECs reserved for this purpose was quantified immunochemically with a commercial enzyme-linked immunosorbent assay kit (American Diagnostica, Greenwich, Conn).[3,4] The supernatants from matched cultures of transduced cells and nontransduced control cells were assayed for urokinase production for the 10 to 12 days immediately after transduction.

Four-millimeter expanded polytetrafluoroethylene (ePTFE) grafts (W.L. Gore and Associates, Flagstaff, Ariz) were lined with transduced ECs with the technique originally described by Kesler et al[5] and subsequently modified in our laboratory to enhance cell attachment.[3,4,6] Before graft seeding was performed, recovery of cells was allowed for 7 days after exposure to G418 to increase cell adherence.[3] EC-lined grafts were incubated 4 days in culture medium after seeding before they were tested in the pulsatile flow apparatus; this incubation period also improved adherence.[3]

Pulsatile flow system. Urokinase-transduced peripheral blood–derived ECs were tested for physical stability of the graft lining under simulated arterial blood flow conditions. Before the graft was connected to the pulsatile flow apparatus, a section was removed for preflow analysis. A 6-cm segment of lined graft was tested in the pulsatile flow system (Harvard Apparatus, South Natick, Mass) pump by the insertion of the linear adaptors into the graft and the binding of the graft to the adaptor with two suture knots and two smooth bulldog clamps at each juncture. Pump settings were 30% output phase ratio (duty cycle), 60 strokes/min, and 10 mL/stroke, which yielded a calculated shear stress of 9.45 dynes/cm^2. The entire flow system was placed in an incubator at 37°C and run for 2 hours. At the end of the flow simulation, the grafts were disconnected and immersed in 10% neutral buffered formalin for fixation. The preflow and postflow graft sections were stained with hematoxylin and analyzed for cell adherence.

Image analysis of graft. Surface characteristics of each graft were evaluated preflow and postflow by computerized image analysis. A high-resolution video image of the hematoxylin-stained graft surface was digitized and stored in memory (Optimas Software, Media Cybernetics, Silver Springs, Md). The number of adherent cells per graft surface area was calculated with a blinded procedure with the operator unaware of the graft history. An average of five repeat, nonsequential analyses of each graft segment were performed. Validation studies of this technique have shown that repeat measures done in a blinded fashion on the same sample typically show a variance of less than 5%.

Table I. Umbilical cord–blood culture growth in various media formulations

Media	*Endothelial cells*	*Line formations*
DMEM + 20% serum + Fe	1/22 (4%)	10/22 (46%)
EC Growth Media + 20% serum + Fe	17/51 (33%)	9/51 (18%)
EC Media (serum free) + bFGF	0/2	0/2
RPMI + 10% serum + Fe + bFGF	0/10	1/10 (10%)
RPMI + 10% serum + Fe	0/3	0/3

Scanning electron microscopy. Samples for scanning electron microscopy were taken from preflow and postflow grafts at several points throughout the interior of the graft. Samples were processed in the standard fashion and examined for cell distribution and cell surface characteristics.

RESULTS

Growth of umbilical cord blood-derived ECs. The effect of matrix on EC development was examined by dividing 19 umbilical cord monocyte samples and culturing each on both gelatin and fibronectin matrices. EC development and line formation were independent of matrix. In the 19 samples, 10 developed EC colonies: four colonies on the fibronectin matrix and six colonies on the gelatin matrix ($P > .5$). In another experiment in which two umbilical cord samples were each cultured on gelatin, fibronectin, and 1:1 gelatin:fibronectin mix as matrix, lines that resembled vasculogenesis formed in all six cultures within the first 7 days; only two of these cultures developed EC colonies (gelatin and 1:1 fibronectin:gelatin matrices). Two milligrams per milliliter of gelatin was used as the matrix for the remaining 42 umbilical cord samples.

The growth of umbilical cord monocyte cultures in various media formulations is summarized in Table I. Cultures grown in EC growth media plus 20% calf serum with iron developed colonies that resembled the typical cobblestone morphologic features of ECs in 17 of 51 (33%) samples. In 1 of 22 (4%) samples that used DMEM plus 20% calf serum with iron,

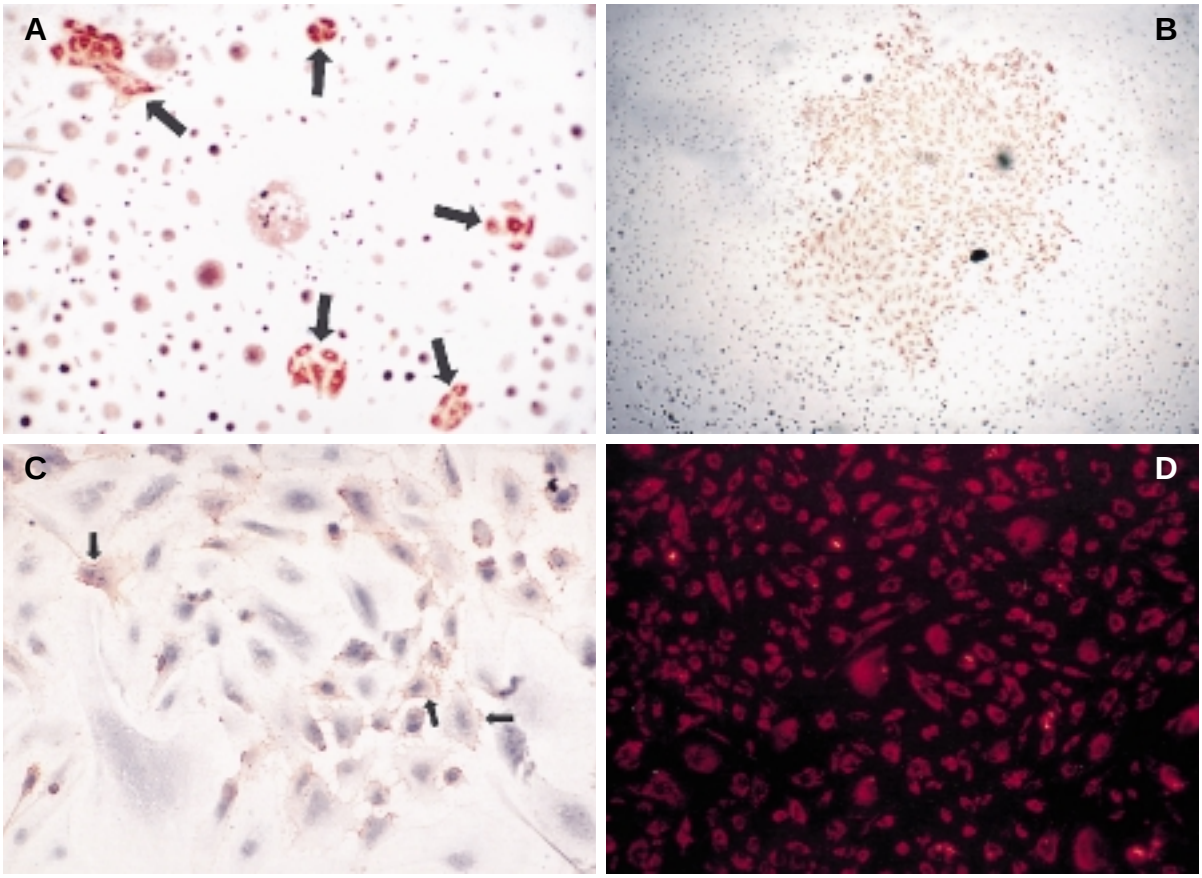

Fig 1. Human umbilical cord monocyte cultures: A, early EC colonies (3-15 cells, *arrows*) with positive staining for factor VIII (*red*), VEGF-R (*blue-black*) staining positively on some surrounding cells (original magnification, ×100); B, later EC colony with positive staining for factor VIII (original magnification, ×40); C, EC staining for CD31 (*red; arrows*) visible on periphery of cells (original magnification, ×200); and D, EC colony identified by uptake of DiI-Ac-LDL (*orange fluorescent*; original magnification, ×100).

colonies resembling ECs developed. Lines that resembled incipient vasculogenesis were seen most frequently in cultures with DMEM plus 20% calf serum with iron. No growth patterns were seen in cultures supported by the other three media solutions.

Initially in umbilical cord monocyte cultures, most of the cells were small and round with many random giant multinucleated cells throughout. Over time, the giant cells decreased in number and became less nucleated, and the smaller cells increased in predominance. Lines resembling the first stages of vasculogenesis appeared as early as 4 days in culture (more commonly, 1-2 weeks). There was great variability in line appearance, from multiple thin strands to thicker tubular-appearing structures. Early lines appeared as a single row of aligned cells, which in some cases further elongated or developed into thickened structures. Complex lines consisted of a double row of elongated cells in contact with each other on the periphery, surrounding a core of almost cuboidal cells up to several cells wide. Line formations usually continued to increase in complexity for up to 3 weeks then gradually became disorganized and disappeared. EC colonies were first visualized at approximately 2 weeks and subsequently increased in size over time. The appearance of line formations did not necessarily predict future EC development, and EC colonies did grow without previous line formations.

Immunostaining for VEGF-R was positive at 1 and 2 weeks for large cells and those involved in line formations. At 3 weeks, VEGF-R staining was positive for cells in EC colonies and line formations. Immunostaining for factor VIII was negative at 1 week for all cells but became positive as soon as small

colonies of ECs were identified (Fig 1, *A*). In some early EC colonies, mixed staining of VEGF-R and factor VIII was observed. Staining for factor VIII increased as the EC colonies grew and remained positive as long as the EC colonies remained in culture up to a maximum of 14 weeks (Fig 1, *B*). Immunostaining for CD31 showed good edge staining of 60% of ECs in defined colonies and continued to be positive up to the 9 weeks these cells were kept in culture (Fig 1, *C*). Fluorescence of internalized DiI-Ac-LDL was positive for colonies with EC morphologic features in culture at 10 weeks (Fig 1, *D*). Human umbilical cord mononuclear cell cultures showed positive staining with anti-human smooth muscle cell actin antibody of an occasional cell in only 1 of 10 cultures from individual cord blood samples tested.

Growth of adult peripheral blood-derived ECs. A mean of $1.44 \pm 0.18 \times 10^5$ cells were isolated in the mononuclear preparation per milliliter of peripheral blood by Ficoll gradient centrifugation. Cultures of adult human peripheral blood that were subjected only to gradient centrifugation to isolate mononuclear cells developed line formations that resembled the initial stages of vasculogenesis in two cultures (Fig 2). No colony growth resembling ECs was identified in these cultures.

Cultures from CD34 magnetic bead–selected adult human peripheral blood cells demonstrated growth of ECs with typical cobblestone morphologic features after 18 days (Fig 3, *A*). Line formations that resembled vasculogenesis (as described earlier for umbilical cord blood cultures) were identified in some cultures from cells not adhering to CD34 magnetic beads ($CD34^-$ cells). No EC colonies were seen in cultures from $CD34^-$ cells. Immunostaining for CD31 on cultures from $CD34^+$ cells with colonies of ECs was positive when tested at 5 and 8 weeks, with the same pattern of edge staining seen in umbilical cord blood cultures. Immunostaining for CD34 after 8 weeks in the cultures from initially $CD34^+$ cells demonstrated 20% staining of cells within EC colonies (Fig 3, *B*). Factor VIII immunostaining was also positive in ECs cultured from $CD34^+$ cells when tested at 5 and 8 weeks (Fig 3, *C*). Two human peripheral blood cultures showed negative immunostaining results when tested with anti-human smooth muscle cell actin.

Urokinase production by transduced ECs and pulsatile flow studies. By 5 days in culture, urokinase levels above background were detectable in transduced cells (Fig 4). At 10 days after transduction, peripheral blood-derived ECs showed production of urokinase at 0.084 ng / mL / hr / 10^6 cells, and umbilical cord–derived ECs at 10 days after transduction showed production of urokinase at 0.154 ng / mL / hr / 10^6 cells. Levels of urokinase in nontransduced umbilical cord–derived cells were not detectable above background; levels in nontransduced peripheral blood–derived cells were 0.015 ng / mL / hr / 10^6 cells ($P > .05$ compared with transduced cells).

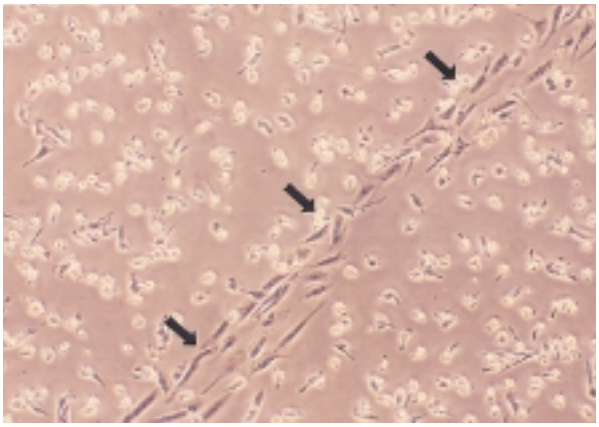

Fig 2. Human adult peripheral monocyte culture (gradient separation only) demonstrates line formation *(arrows)* that resembles vasculogenesis. (Unstained; original magnification, ×100.)

In two experiments that used transduced adult peripheral blood–derived cells to line 4-mm ePTFE grafts, the mean postflow retention of cells was 87.33% ± 4.97% of the number of preflow cells, a change that was not significant by the Student *t* test ($P > .05$). Similar levels of attachment were seen in two experiments with transduced umbilical cord–derived cells, in which a comparison of preflow and postflow cell layers showed retention of 82.94% ± 25.95% of cells. Light microscope and scanning electron microscope examination of the postflow graft segments in these experiments showed mostly even distribution of well-attached cells with typical spindle-shaped or rounded EC appearance. Areas of cell loss showed nondistinct cell boundaries or roughened attachment. Occasional bare patches found in both preflow and postflow grafts represent lack of full coverage in seeding rather than detachment because of shear stress.

DISCUSSION

The purpose of this study was to develop a method for obtaining EC precursors from human blood to be used as a source of ECs for seeding pros-

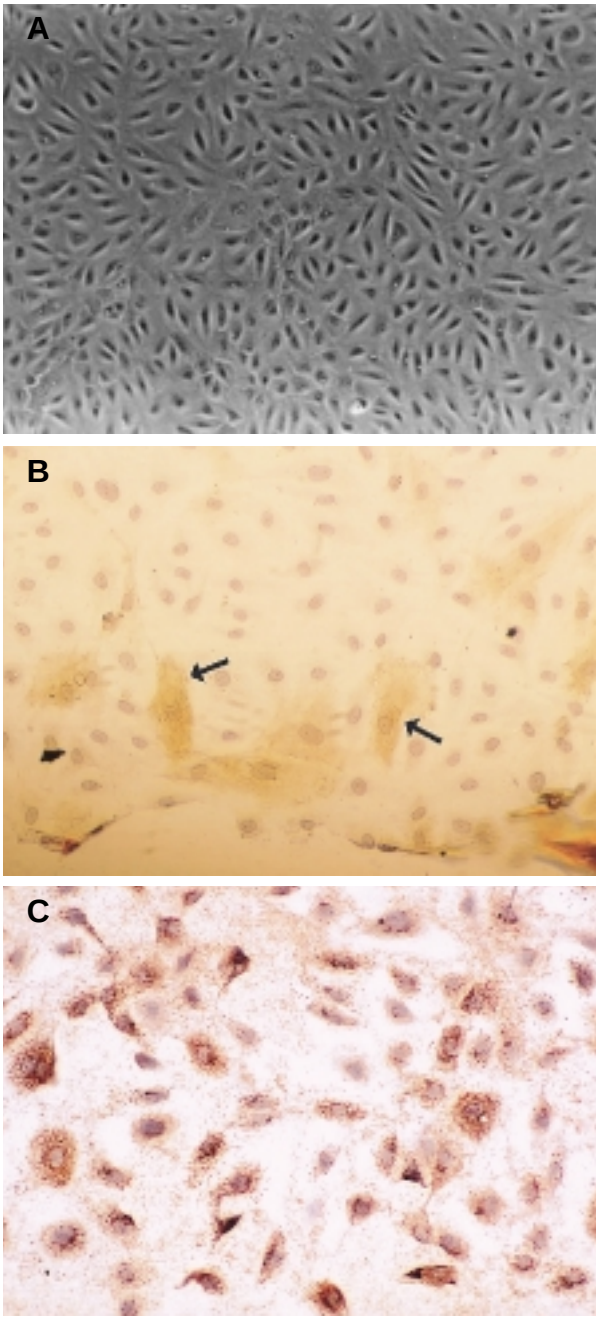

Fig 3. Human peripheral blood cultures (magnetic bead separation): A, $CD34^+$ cell fraction shows colony with typical EC morphologic features (unstained; original magnification, ×100); B, CD34 staining of initially $CD34^+$ ECs, note random staining pattern (*brown, arrows;* original magnification, ×200); and C, factor VIII staining (*red, granular*) of $CD34^+$ EC (original magnification, ×200).

thetic vascular grafts and for use in gene therapy. Human prosthetic vascular grafts infrequently form an endothelial layer on the lumenal side unlike canine, bovine, and porcine animal models, although Berger et al[7] showed in 1972 that humans have ingrowth of approximately 2 to 4 mm from both the proximal and distal anastomoses, never exceeding 10 mm; Shi et al[8] identified endothelialized patches in some human long-term Dacron grafts that had been fixed immediately after death. To answer the question as to whether animals endothelialize grafts by ingrowth or whether ECs or EC progenitor cells are circulating in peripheral blood, Stump et al[9] provided evidence for the presence of ECs in the peripheral blood by observing canine ECs adherent on an intravascular Dacron hub. ECs were shown to exist in peripheral blood by Scott et al,[10] possibly as progenitor cells. Fallout endothelialization was shown to occur in the middle section of the lumen of canine vascular grafts in small patches with experimental Dacron-ePTFE-rubber vascular grafts that prevented EC migration through the wall of the vascular graft; anastomotic pannus ingrowth did not extend beyond 13 mm in any specimen in this study.[11] More recently, with the same type of graft, the middle section ECs were shown to be derived from the bone marrow, suggestive of EC progenitor cell fallout endothelialization from the blood.[12] These studies suggest that ECs and/or their progenitor cells exist in peripheral blood of both humans and animals.

Other investigators have studied hematopoietic progenitor cells from adult peripheral blood and from umbilical cord blood. Blood stem cell pools and bone marrow are in dynamic equilibrium with each other, thus allowing migration of cells between extravascular marrow sites and the circulation.[13] Cord blood contains 20 times the number of clonogenic progenitor cells than adult peripheral blood.[14] Recently, methods have been developed for ex vivo hematopoietic expansion[15] and isolation and purification techniques for desired cell populations.[16,17] Much of this work relates to the isolation and in vitro development of ECs.[18]

Studies in the field of vasculogenesis show that the hemangioblast is a common precursor for both hematopoietic cells and ECs; the hemangioblast differentiates into (1) hematopoietic stem cells and (2) EC precursor cells.[19,20] CD34 is the common surface marker for cells of the hematopoietic lineage but is also found on capillary ECs, less on larger vessels,[21] and in a posttranslationally processed form in the high endothelium of the postcapillary venules of lymph nodes.[22] CD34 expression is downregulated in mature ECs in culture.[21] EC surface receptors that appear early in development are VEGF recep-

tor-1 and -2, vascular endothelial cadherin (also known as cadherin-5), platelet endothelial cell adhesion molecule-1 (CD31), tie-1, tie-2, and CD34.[22] There are two tyrosine kinase receptors for VEGF expressed on the surface of various ECs, flk-1 and flt-1. In normal development, VEGF receptors appear to be expressed during vasculogenesis and early vascular differentiation.[23] Consistent with this, we find VEGF-R staining in line formations and in early EC colonies. The development of lines that stain positively for VEGF-R, well before EC can be identified in culture, suggest that these lines are being formed by progenitor ECs. In early EC colonies, immunostaining for CD31 showed good edge staining that was consistent with its concentration at cell borders where it mediates cell-cell adhesion and transduces intracellular signals.[24,25] Cells in our cultures develop increasing levels of factor VIII over time; it has been reported that mature ECs in culture may lose factor VIII expression as cells approach senescence.[26,27] The progression of cell types and surface markers seen in our cultures is strongly suggestive of the development of EC precursors.

In these studies, inclusion of mature ECs released from vessel walls cannot be entirely ruled out; however, steps were taken to minimize the handling of the vessels, and the initial portion of each peripheral blood draw was discarded specifically to exclude this possibility. Previous experience in our laboratory with cultures of mature vascular endothelial cells has shown that ECs from vessel walls are less fragile and show stronger attachment to the matrix than progenitor cells. Especially in umbilical cord samples, mature ECs plate down immediately, grow very rapidly, and never show the migration of cells into lines resembling the first stages of vasculogenesis. ECs in this study developed over 18 days in culture and were not identifiable as ECs until that time, further suggesting that they were not present as mature ECs in the blood collected, but developed from precursor stem cells.

Isolation of ECs and progenitors from human cord blood led us to the study of adult human peripheral blood. Gradient centrifugation alone with culture of the resulting monocyte fraction produced lines of cells that appeared more developed than those in the CD34$^-$ fraction, probably because of growth factors or matrix components produced by cells that are removed during magnetic bead separation. These lines are quite similar to incipient vasculogenesis as reported by Risau and Flamme.[28] Several growth factors have been identified with vasculogenesis, including VEGF, bFGF, heparin, and the undefined endothelial cell growth supplement and tumor-conditioned culture supernatant.[28]

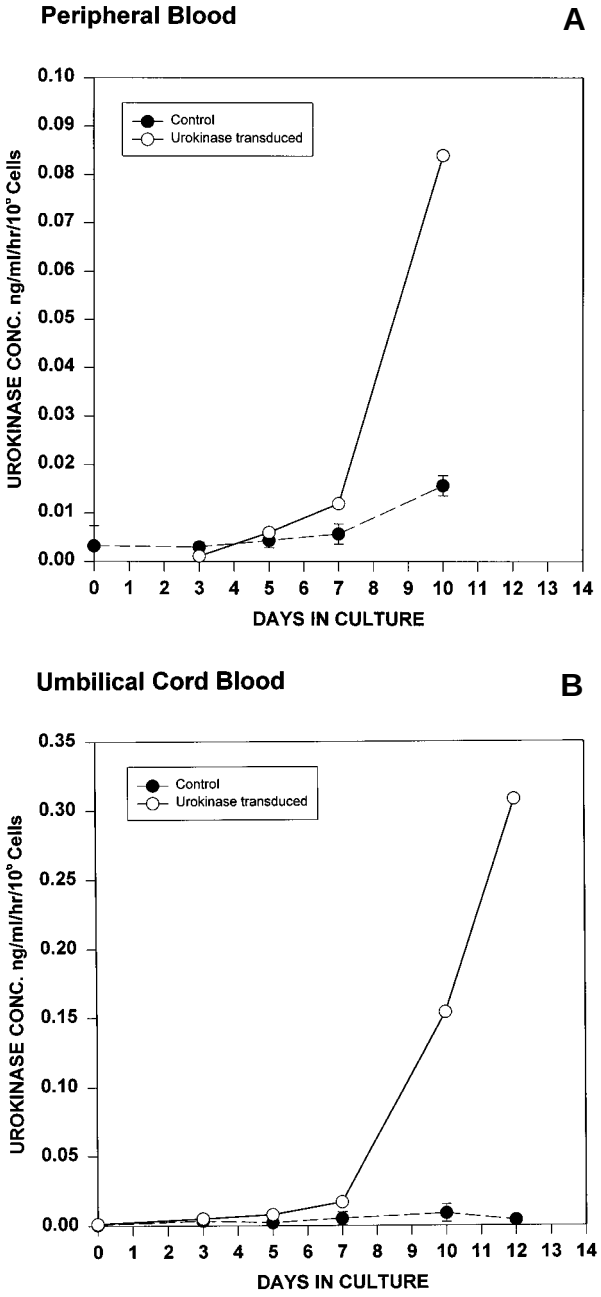

Fig 4. Overexpression of urokinase by genetically modified peripheral blood–derived endothelial cells (A) and umbilical cord–derived endothelial cells (B). Urokinase production quantified by enzyme-linked immunosorbent assay of culture supernatants over 10 and 12 days after transduction with a retroviral vector containing the gene for human prourokinase. Control medium is culture supernatants from matched nontransduced cells.

Similar work to ours has been recently reported by Asahara et al[29] who described separation of EC progenitors only from adult human peripheral blood by the magnetic bead method. They found fully differentiated ECs after 7 days in culture, compared with 18 days for our adult peripheral blood cultures. Their results also differ from ours in that they found lines of cells, somewhat dissimilar to ours, in the $CD34^+$ fraction. We found such line formations in the $CD34^-$ fraction or the cultured unfractionated monocyte preparation, which shows the most complex lines of cells. The lack of correlation between line formation and EC colony development in $CD34^+$ and $CD34^-$ fractions may indicate that different populations of progenitor cells are involved or that the growth factors required for differentiation and/or vasculogenesis are not produced in the $CD34^+$ fractionated population. Additionally, Lindner et al[30] have suggested that activated mononuclear cells may secrete substances inhibitory or toxic to EC colony development, but which do not inhibit the incipient vasculogenesis seen in our cultures. Further refinements in growth conditions presented to unfractionated mononuclear cell preparations or the $CD34^-$ fraction (such as the addition of growth factors, use of conditioned medium, alterations in feeding schedule, mechanical removal of cells or the specific isolation of progenitor cells) may enhance the development of any EC precursors found in these populations. The use of a thicker matrix may encourage the development of true tubular structures from the line formations we have observed.

In previous studies, we transduced canine EC derived from surgically harvested jugular vein with the gene for prourokinase and used these cells to line prosthetic ePTFE grafts.[3] In this study we have shown similar transduction of human umbilical cord and peripheral blood–derived cells. With the use of the culture techniques described, these cells remain more adherent under clinically relevant pulsatile flow conditions in vitro than have been reported in other studies for transduced cells producing tissue plasminogen activator.[31] ECs cultured by these methods show potential for prosthetic graft applications, although in vivo testing in humans remains to be done.

In conclusion, these studies indicate that human peripheral blood may provide a reliable source of endothelial cells for graft seeding and gene therapy, and this technique combined with methods of tissue engineering has the potential to produce a small diameter vessel. These are areas that warrant further investigation.

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